Spring 5-22-2015

Mechanisms of axial polarity modification during postembryonic development of the basal bilaterian Convolutriloba macropyga

Laura N. Crowther
University of San Francisco, lncrowther@dons.usfca.edu

James M. Sikes Ph.D.
University of San Francisco, jsikes@usfca.edu

Mechanisms of axial polarity modification during postembryonic development of the basal bilaterian *Convolutriloba macropyga*

Laura N. Crowther

Department of Biology, College of Arts and Sciences, University of San Francisco, San Francisco, CA

Research Advisor:
James Sikes, PhD

Department of Biology, College of Arts and Sciences, University of San Francisco, San Francisco, CA

May 2015

Table of Contents:

Abstract	3
Introduction	5
Results	8
Pharmacological Inhibition Trials	8
qPCR	10
RNAi	11
Discussion	12
Methods	16
Animal Culture	16
Pharmacological Inhibition Trials	16
qPCR	16
Cloning	17
RNAi Trials	18
Phalloidin Staining	19
Microscopy and Imaging	19
Works Cited	20

Abstract

Acoel flatworms have varied modes of asexual reproduction that involve dramatic postembryonic modification of their anterior-posterior (AP) axis. The acoel species *Convolutriloba macropyga* reproduces through a reversed polarity budding process in which offspring develop from two posterior budding sites with a complete reversal of the AP axis compared to the parent. Reversed polarity budding is preceded by the development of a zone of tissue with disorganized musculature that is incapable of regeneration, suggesting a transient loss of axis polarity at each budding site. For this reason, these tissues are titled the polarity transition zone (PTZ). While this alteration of existing axial polarity seems to be required for subsequent reversal of the AP axis in the budding progeny, the mechanisms that allow for temporary axis modification and reversal are not known. Wnt, Hedgehog, and other signal transduction pathways have conserved roles in AP axis development and reestablishment during both metazoan embryogenesis and regeneration, suggesting these signals may function in mediating changes in axis polarity during budding in *C. macropyga*. Here, we have used a pharmacological screen to perturb conserved signaling pathways in *Convolutriloba* tissues and observed loss of axial polarity in tissues exposed to inhibitors of the Hedgehog pathway. Given the putative role of Hedgehog signal transduction in mediating alterations in axial polarity during budding, we have quantified changes in the expression of Hedgehog signaling components and regulators in budding tissues using qPCR. Hedgehog signaling was downregulated within the PTZ when compared to neighboring polarized tissues. RNAi mediated knockdown of Hedgehog gene products resulted in phenotypes of delayed bud detachment and failure to initiate future budding events. These data support Hedgehog as a key signaling pathway

involved in the modification of AP axis polarity during asexual reproduction and may provide key insights towards better understanding the evolution of asexual reproduction strategies in other taxa.

Introduction

The establishment of body axis polarity is a critically important step in bilaterian development serving to create a coordinate system that mediates subsequent developmental processes during embryogenesis (Nusslein-Volhard & Weischaus 1980; Martindale 2005). For the majority of bilaterian animals, once axial patterns have been established they remain unchanged throughout the life of the organism (Caroll *et al.* 2005; Martindale 2005). However, some animals can modify their existing axes or develop novel body axes during postembryonic events. During regeneration, axes must be reestablished prior to the restoration of lost or damaged body parts (Liu *et al.* 2013; Sikes & Newmark 2013). Asexual reproductive events such as budding and fission involve modifications of existing axes or formation of novel axes (Hughes 1989; Sikes & Bely 2008). The most radical modification of axial polarity occurs during reversed polarity budding when the anterior-posterior (AP) axis undergoes localized reversal to produce axes with opposite orientation (Hendelberg & Akesson 1988; Hendelberg & Akesson 1991).

Two acoel species, *Convolutriloba retrogemma* and *Convolutriloba macropyga*, are unique in their ability to undergo reversed polarity budding in which a bud develops along the posterior margin with AP polarity completely reversed relative to the parent (Shannon & Achatz 2007; Sikes & Bely 2010) (Figure 1). A region of tissue in the polarity transition zone (PTZ) between parent and bud where AP axis reversal presumably occurs is characterized by disorganized musculature (Sikes & Bely 2008) (Figure 2). Tissues excised from this zone fail to regenerate and become radialized, unlike tissues taken from the parent and bud regions (Figure 2). Lack of polarity has been shown to inhibit regenerative

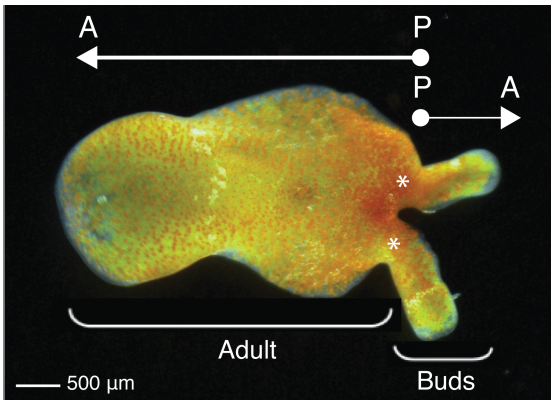

Figure 1. Reversed polarity budding results in progeny that possess an AP axis directionally opposed to the parent. A – Anterior, P – Posterior.

abilities in other flatworms (Liu *et al.* 2013; Sikes & Newmark 2013; Umesono *et al.* 2013), suggesting that these tissues may lack polarity. These data suggest that the formation of a region lacking polarity presumably occurs prior to bud development and AP axis reversal. This study seeks to identify the underlying mechanisms that modulate this axis modification in the PTZ.

Conserved signal transduction pathways are important in establishing axial polarity in both embryogenesis and regeneration (Petersen & Reddien 2009). The interaction of different signaling pathways directs the polarity reestablishment that must occur following amputation. For example, the interplay of Extracellular-signal Related Kinase (ERK) and Wnt pattern the AP axis during regeneration in planarians (Gurley *et al*. 2008; Iglesias *et al*. 2008; Peterson & Reddien 2008; Rink *et al*. 2009; Yazawa *et al.* 2009; Gurley *et al*. 2010; Umesono *et al.* 2013). In addition, Hedgehog signals have been shown to modulate AP polarity by directly affecting the signals of Wnt and other signaling pathways (Gurley *et al*. 2008; Iglesias *et al*. 2008; Peterson & Reddien 2008; Rink *et al*. 2009; Yazawa *et al.* 2009; Gurley *et al*. 2010). While there have been extensive efforts to understand these signals in regeneration, the molecular mechanisms that modulate postembryonic axis development during asexual reproduction have received little attention. *Convolutriloba* acoels offer a unique opportunity to investigate the molecular mechanisms that modify axis polarity during asexual reproductive events. Understanding the role of conserved signal

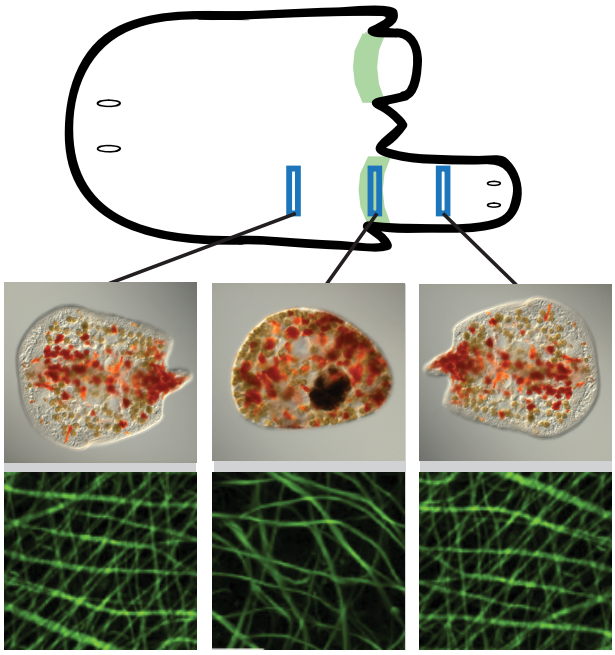

Figure 2. Tissues from the PTZ display disorganized body wall musculature and fail to regenerate. Muscle fibers are visualized via phalloidin staining. Tissues excised from parental and bud regions regenerate distinct AP polarity.

transduction in the development and propagation of the PTZ will provide insights into *in vivo* axis modification. In this study, we seek to identify the molecular signals responsible for the loss of polarity in the PTZ by using chemical genetics to perturb candidate pathways. We find evidence that Hedgehog signal transduction may be modulating this process and perform further investigations to validate the role of Hedgehog.

Results

Pharmacological Inhibition Trials

Given conserved roles in AP axis development during embryogenesis and regeneration, Wnt and Hedgehog signaling pathways are primary candidates that may play a role in the loss of polarity in the PTZ. In order to determine if either pathway is modulating the processes responsible for development of apolar tissue, we conducted a chemical genetic screen in *C. retrogemma*. To perturb Wnt signaling we used the following pharmacological agents: IWR-1-endo and XAV939 as inhibitor agents and QS11 as a synergistic agent. IWR-1-endo and XAV939 both directly target Axin, which negatively regulates Beta-catenin transcriptional control (Huang *et al.* 2009). Alternatively, QS11 targets the Wnt-3a ligand

activating canonical Wnt signaling (Zhang *et al.* 2007). In addition, we used other drugs to perturb Hedgehog signaling: GANT61 and Cyclopamine inhibit Hh signaling while Purmorphamine acts as a synergistic agent. GANT61 and Cyclopamine both negatively influence Gli's ability to regulate transcriptional activity (Chen *et al.* 2002; Hyman *et al.* 2009; Wang *et al.* 2009) while Purmorphamine activates Hh signaling by acting as an agonist towards Smoothened, a positive regulator (Sinha & Chen 2006).

We did not observe a phenotype when animals were exposed to either inhibitors or synergists of the Wnt pathway. All animals maintained a distinct anterior with eyespots and a distinct posterior tri-lobed tail similar to DMSO controls. Contrastingly, when animals were exposed to Hedgehog inhibitors GANT61 and Cyclopamine the animals lost polarity and took on a radialized appearance similar to excised PTZ tissues that failed to regenerate. However, animals exposed to the Hedgehog synergist Purmorphamine maintain visible anterior and posterior morphologies (Figure 3). To determine if affected animals phenocopy the body wall musculature of the PTZ, we stained animals exposed to Hedgehog inhibitors with phalloidin (Figure 4). Actin filaments failed to demonstrate the highly organized pattern typical of adult body tissues suggesting a potential loss of axial polarity within all tissues of the animal. Together these data suggest that upregulation of Hedgehog signaling is important for the maintenance of axial polarity within *Convolutriloba* tissues.

qPCR

Considering the putative role of Hedgehog signaling in the maintenance of axial polarity in *Convolutriloba*, we aimed to quantify the expression levels of Hedgehog related genes

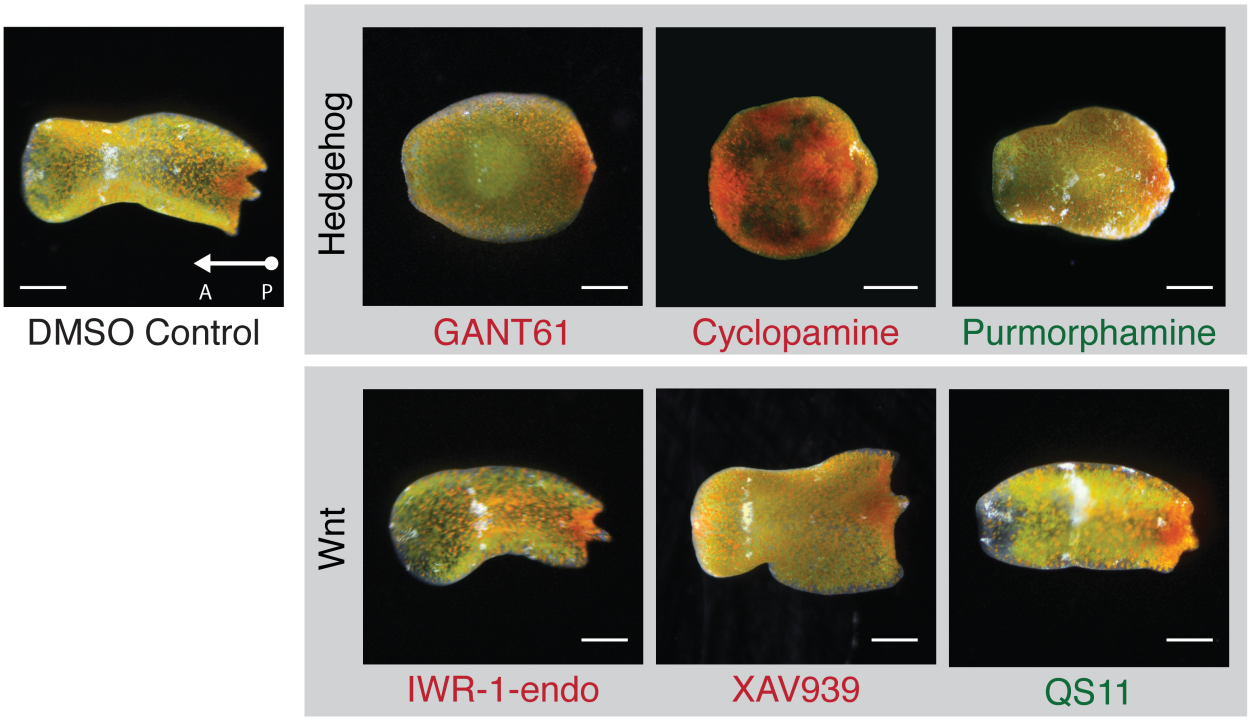

Figure 3. Adult *C. retrogemma* were exposed to pharmacological inhibitors (red) or synergists (green) of the Wnt and Hedgehog pathways. GANT61 and Cyclopamine treated animals lost polarity and started to radialize. Perturbation of Wnt signaling resulted in no alteration of body pattern. Scale bar, 500 μm.

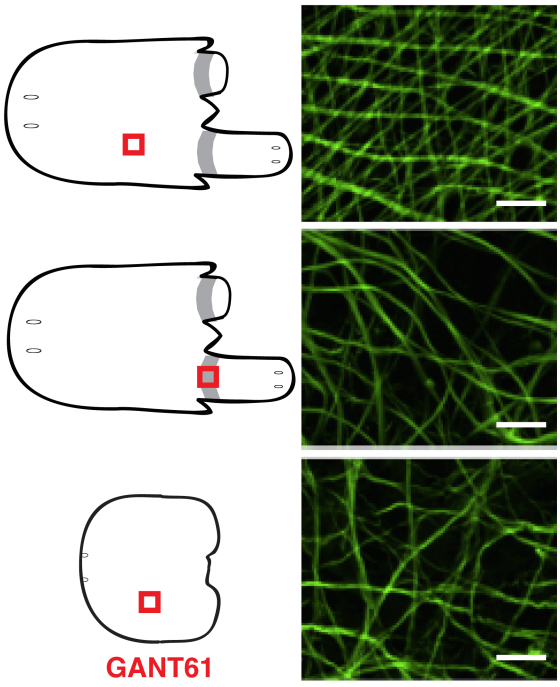

Figure 4. Body wall musculature is modified in both the PTZ and in animals treated with Hedgehog inhibitor GANT61. Scale bar, 15 μm.

within the PTZ. We developed three tissue-specific cDNA libraries from tissues excised from polarized adult body regions, polarized bud regions, and tissues from the PTZ (Figure 5). Hedgehog signaling is a complex interaction of multiple proteins including a ligand, two transmembrane receptors, intracellular modulators, and an intracellular transcription effector (Figure 6). We chose to quantify a subset of Hedgehog related genes including a

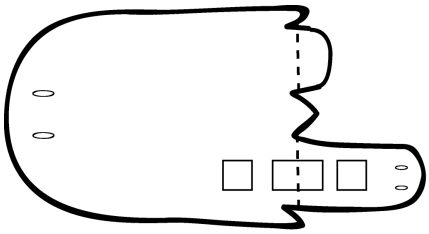

Figure 5: cDNA synthesized from three tissue regions were used in the qPCR trials: the parental region (left square), the PTZ (middle square), and the bud region (right square).

ligand (*hedgehog*), a receptor (*patched*), related intracellular molecules (*fused* and *costal2)*, and the direct effector of transcription (*gli*). Relative to parental tissues, *hedgehog* ligand expression is downregulated in the PTZ while all other candidate genes are upregulated in the PTZ and the developing bud (Figure 7). In contrast, the *hedgehog* ligand is upregulated in the bud compared to the parental tissues. These data suggest that Hedgehog signaling is downregulated in apolar tissue regions but signaling appears active in tissues where polarity is present.

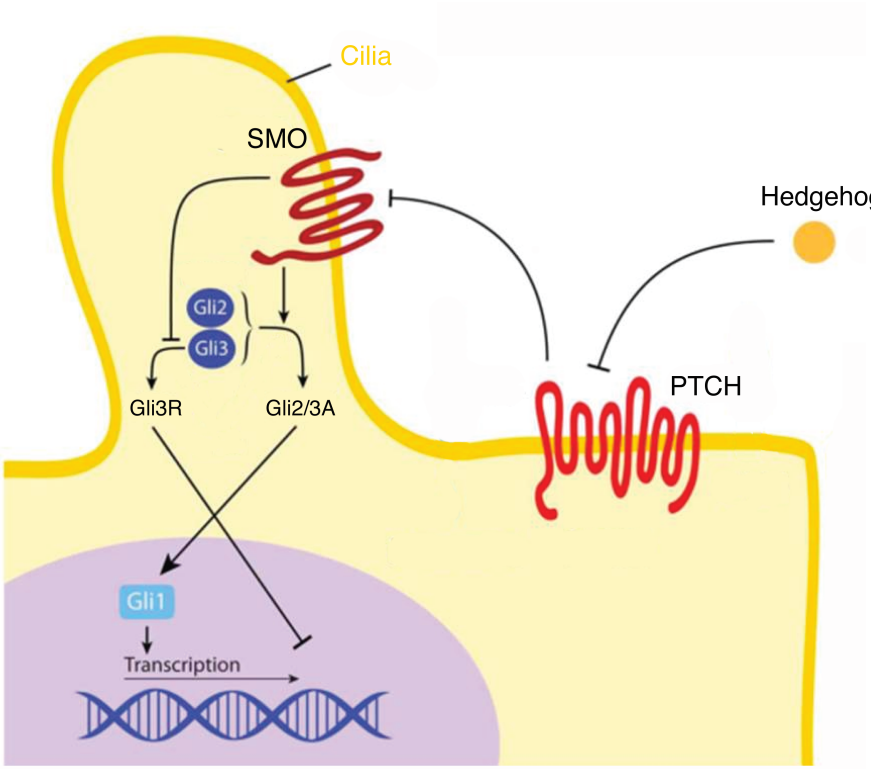

Figure 6: Candidate Hedgehog signaling genes targeted for qualitative analysis. Modified from Pan *et al.* 2013.

RNAi

While chemical genetic data suggest Hedgehog signaling modulates polarity in adult tissues of *Convolutriloba*, off-target effects from administrating drug treatments are possible,

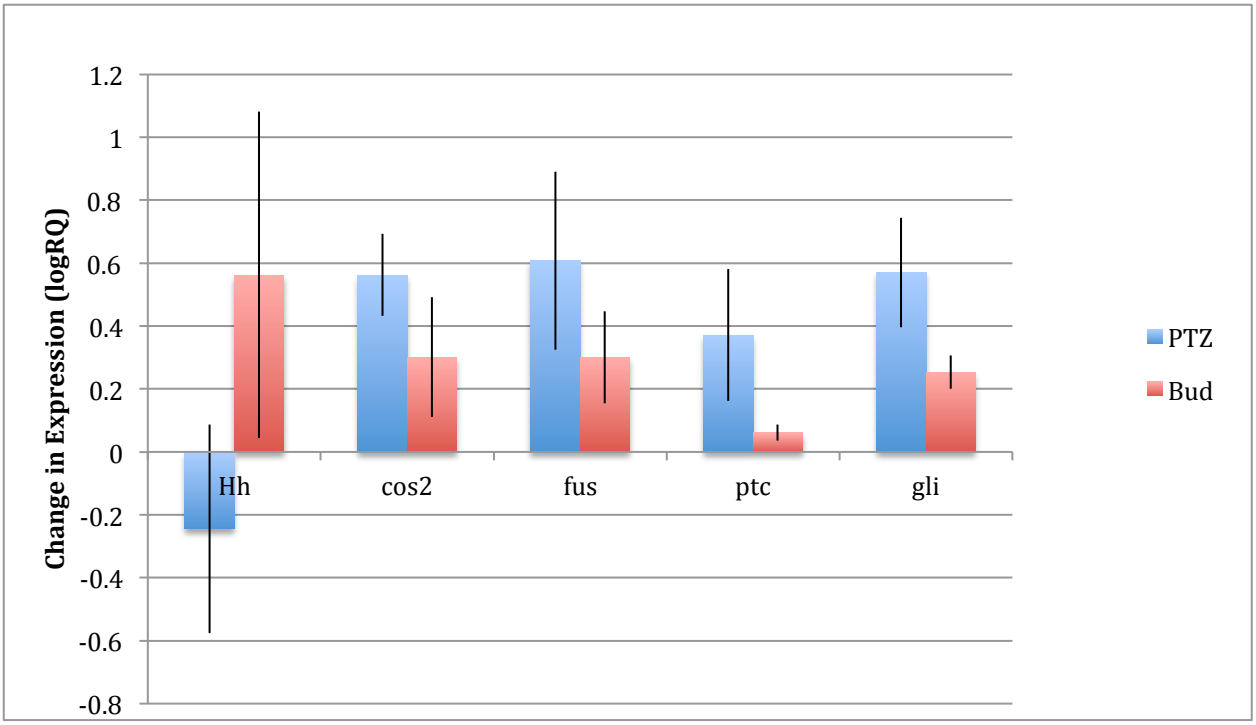

Figure 7: Log change in gene expression in the bud tissues and the PTZ tissues standardized to parental tissues. Error bars represent standard deviations.

potentially producing phenotypes not directly resulting from perturbation of Hedgehog signaling. In order to confirm the role of Hedgehog signaling in polarity maintenance and loss during budding in *Convolutriloba,* we conducted RNAi mediated gene knockdown of specific Hedgehog signaling genes. Following microinjection of dsRNA into the gut of budding *C. macropyga*, we made a qualitative assessment of alterations to the budding process. RNAi experiments to knockdown *fused, hedgehog,* or *gli* each resulted in delayed bud detachment or lack of budding phenotype relative to control RNAi (data not shown). While these data were not quantitative in nature, these results suggest Hedgehog signaling may play a role in maintaining the PTZ and the subsequent initiation of future budding events.

Discussion

Few animals have the potential to modify or manipulate preexisting axial polarity. The mechanisms allowing for this modification are central to understanding the evolution and development of diverse asexual reproduction strategies. We have further developed *Convolutriloba* acoels as models to elucidate the molecular signals that mediate changes in axis polarity *in vivo* during reversed polarity budding. While budding in *Convolutriloba* appears as linked reversed AP axes, a region of apolar tissue termed the polarity transition zone separates the two juxtaposed axes. This study characterizes Hedgehog signaling as a key regulator in the maintenance and loss of polarity that drives reversed polarity budding. Hedgehog has been shown in planarians to affect the reestablishment of AP axis polarity by directly modulating Wnt expression (Rink *et al*. 2009). Given these data and the highly conserved role of Wnt signal transduction in the development of AP axial polarity during embryogenesis and regenerative outgrowth (Martindale 2005), we expected Wnt signaling to play a key role in reversed polarity budding. However, perturbation of Wnt signaling had no effect on axial polarity. Acoels occupy a unique phylogenetic position basal to all other bilaterians (Ruiz-Trillo *et al.* 1999; Paps *et al*. 2009), suggesting a possible pleseomorphic condition in which AP axes may be patterned in the absence of Wnt signaling. Yet, Wnt signaling controls regeneration of the AP axis in *Hofstenia miamia*, a derived acoel species (Srivastava *et al.* 2014). While our data do not implicate Wnt signaling, the nature of chemical genetic screens, and mode of action of specific drugs limit our attempts to definitively identify all candidate signaling mechanisms. Targeted RNAi of Wnt components may demonstrate a role of Wnt signaling in this process.

The expression of *hedgehog* is downregulated in tissues lacking polarity suggesting that Hedgehog signaling maintains polarity within *Convolutriloba* tissues. While quantitative analysis of the expression of other Hedgehog signaling genes shows increases in expression in both the PTZ and budding tissues, the upregulation of the *hedgehog* ligand is requisite for Hedgehog signaling. A downregulation of *hedgehog* alone correlates to a reduction in signaling that potentially mediates a transient and spatially restricted loss of polarity within the PTZ. Significant upregulation of Hedgehog signaling in the bud is not surprising given the active developmental processes that are patterning asexual progeny.

The body-wide loss of polarity resulting from pharmacological inhibition is not phenocopied by gene-targeted knockdown of specific Hedgehog signaling components. However, initiation of subsequent budding events and modifications of the timing of bud detachment seem to be affected when Hedgehog signaling is downregulated by RNAi. The absence of subsequent budding events upon Hedgehog knockdown is likely a result of failed axis development given the upregulation of the increase in *hedgehog* expression in actively developing buds. The temporal delay in bud detachment may be due to a loss of the PTZ due to changes in Hedgehog signals. Without a localized PTZ with disorganized muscle fibers, characteristic tearing of tissue is likely to be hindered thus delaying bud detachment. These data suggest that Hedgehog knockdown limits the presence of a PTZ, which seems to contradict both the chemical genetics data and the changes in Hedgehog signaling expression levels assayed by qPCR. Given the qualitative nature of the data, we need to quantify the timing of bud detachment in both control and RNAi treated animals to determine the extent to which bud detachment is delayed. In addition, phalloidin staining

of RNAi treated animals would determine if the pattern of muscle organization is altered from that of a normal PTZ. This would provide further evidence for elucidating the function of each gene product.

While this study implicates Hedgehog signaling, alteration in the transcription of countless other genes may mediate *in vivo* changes in axial polarity in addition to Hedgehog signals. A forward genetics approach utilizing RNAseq to compare transcriptomes of polarized tissue and the PTZ will identify candidates differentially expressed within these tissues. In addition, further RNA sequencing of Hedgehog RNAi samples compared to control RNAi samples would identify potential downstream targets of Hedgehog signaling that may mediate the polarity modifications that we have described.

Given previously limited data on polarity modifications during asexual reproductive events, this study suggests that Hedgehog is important for *in vivo* modification of already existing axes. Prior studies have implicated Wnt in regenerative and embryonic patterning events, but this study provides the first evidence for the modification and even loss of axial polarity due to tissue-specific alterations of paracrine signaling. Reversed polarity budding represents one of the most radical postembryonic modifications of axial polarity that seems to be mediated by Hedgehog signaling. Potential alterations of Hedgehog or similar signals in polarized tissue may mediate modifications that could alter the potential for postembryonic development in a variety of animal taxa. Harnessing the ability to alter tissue polarity *in vivo* has unique potential to one day allow for the re-initiation of development in the adult soma, a critical first step in regenerative medicine.

Methods

Animal Culture

Acoels were cultured as isogenic lines (originally established from a single individual) in 12 gallon aquaria at 24°C with a 12 h:12 h light:dark cycle in 34ppt artificial seawater (ASW, Instant Ocean). *Artemia nauplii* were provided weekly as food.

Pharmacological Inhibition Trials

All pharmacological reagents were obtained from Santa Cruz Biotechnology. Stocks of all drugs were made in DMSO and then diluted in ASW to working concentrations of 1-2 μM. Control animals were exposed only to DMSO and ASW. Water and drugs were changed daily. After four days of soaking, polarity loss was observed in the animals exposed to pharmacological reagents. Phenotypes were scored by a loss of the trilobe tail in the posterior region, a loss of eyespots in the anterior region, and the presence of radialized morphology.

qPCR

To examine transcript levels across the budding region, RNA was extracted using TRIzol (Ambion) reagent and Direct-zol RNA Miniprep kit (Zymo) from ten tissue fragments excised from the parent, PTZ, and bud. Following DNase treatment (DNA-free RNA Kit, Zymo Research), reverse transcription was performed using Postscript II Reverse Transcriptase (New England Biolabs)and quantitative PCR was conducted using GoTaq

SYBRgreen qPCR mastermix (Promega) and a CFX96 (Biorad). Two biological replicates were performed and all samples were measured in triplicate to account for pipetting error. Absolute quantities of each transcript were determined for each gene and normalized to the level of GAPDH in each sample. The mean value for each amputated treatment was then normalized to the parental tissue fragments. Primers used for qPCR are included in Table 1.

Table 1. Oligonucleotides used to amplify *C. Macropyga* cDNA and to conduct qPCR

Gene	**Forward Primer**	**Reverse Primer**
cm-Hh	CCCGTCGTTGTACATTGGTCCC	ACAGGGGGATTTTTCAGGGTGCA
cm-cos2	ACGCAACTGTGGACCAAATC	TCATTGCACTGGTGTTGCAC
cm-fus	AGGCGCGAGATCGAGATCATGAA	CGGCAGAGTTCCATCATCCTCCA
cm-ptc	TTGTAAACGACGGCCAGATC	TTGACCGTGCTGACAATCTG
cm-gli	AAAGCGTTCACGGATTGCAC	GGATGTTGTTGGTCGGTGTTG
cm-GAPDH	AACAGCGATTGCAGTTCACG	AGTAGTGAAAACGCCAGTCG

Cloning

For cDNA preparations, RNA was extracted using TRIzol Reagent (Ambion). Candidate genes were PCR amplified from cDNA generated from total RNA using Postscript II Reverse Transcriptase (New England Biolabs). For cloning, 2–3 µl of PCR product was ligated with 70ng of Eam1105I-digested pJC53.2 (Collins *et al.* 2010) using T4 DNA ligase (New England Biolabs) and used to transform JM109 Escherichia coli cells. All primers to amplify candidate genes were designed based on a previously sequenced *C. macropyga* assembly and are included in Table 2.

Table 2. Oligonucleotides used to amplify *C. Macropyga* cDNA and in the cloning procedure.

Gene	Forward Primer	Reverse Primer
cm-Hh	CCAGAGACAGGGGGATTTTTCA	GGGCCTCTTCGCATTAATTAGC
cm-cos2	ATTTGGCCCCAGTAGTTCTCAG	GTTGCATACACTTCTCCTGGGA
cm-fus	TTTATGGTCTCTGGGCTGCATT	TCTGTTGAAGCATGATCGGTGA
cm-gli	ATTCACTGGAGGGTACGAACAC	TGTTTAGCCCTGTCTGATGCAT

RNAi trials

To generate dsRNA, templates cloned into pJC53.2 (Collins *et al.* 2010) were amplified with a modified T7 oligonucleotide (GGATCCTAATACGACTCACTATAGGG), purified using a DNA Clean & Concentrator kit (Zymo Research), and eluted in 15 µl of water. 10.5 µl of each PCR product was used as template for *in vitro* transcription in a reaction containing 5 µl 100 mM mix of ribonucleotide triphosphates (rNTPs) (Promega), 1 µl high-yield transcription buffer (0.4 M Tris pH 8.0, 0.1 M MgCl2, 20 mM spermidine, 0.1 M DTT), 1 µl thermostable inorganic pyrophosphatase (New England Biolabs), 0.5 µl RNAse in Plus RNAse Inhibitor (Promega), and 2 µl T7 RNA polymerase. Samples were incubated at 37°C for 12 h and then treated with RNase-free DNase and cleaned/concentrated via ammonium acetate precipitation. Synthesized RNA was then annealed by heating at 95 °C, 75°C and 50°C each for 3 min. dsRNA solution was mixed with dye and 65 nl (~1µg/µL) was microinjected into the gut of midstage budding adult *C. macropyga* four times over the course of 1 week using a Nanoject II micromanipulator (Drummond Scientific). As a negative control, animals were injected with dsRNA synthesized from the ccdB- and camR-containing insert of pJC53.2 (Collins *et al.* 2010).

Phalloidin Staining

Worms were relaxed in 4% formaldehyde in artificial seawater for 30 min, washed at least three times in PBS, washed in PBTx (PBS+0.1% Triton X), incubated with Alexa Fluor-488 phalloidin (Invitrogen) at 1:100 in PBTx for 2 hours, and then washed three times in PBS. Samples were mounted in Vectashield (Vector Labs).

Microscopy and Imaging

Samples from the pharmacological trials were imaged with a Nikon SMZ-745T stereomicroscope equipped with a Nikon DS-Fi2 camera interfaced through ViewNX2 (Nikon). Phalloidin samples were imaged using a Zeiss LSM-500 confocal laser-scanning microscope using the Zen Black edition (Zeiss). Image processing was performed with Adobe Photoshop CS6 (v. 13.0).

Works Cited

Carroll S, Grenier J, Weatherbee S (2005). *From DNA to Diversity: Molecular Genetics and the Evolution of Animal Design.* Blackwell.

Chen JK, Taipale J, Cooper MK, Beachy PA (2002). Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes & Development* 16:2743-2748.

Collins JJ, Hou X, Romanova EV, Lambrus BG, Miller CM, Saberi A, Sweedler JV, Newmark PA (2010). Genome-wide analysis reveals a role for peptide hormones in regulating planarian germ line development. *PLoS Biology* 8.

Gurley KA, Rink JC, Sanchez-Alvarado A (2008). Beta-catenin defines head versus tail identity during planarian regeneration and homeostasis. *Science* 319:323-327.

Gurley KA, Elliott SA, Simakov O, Schmidt HA, Holstein TW, Sanchez-Alvarado A (2010). Expression of secreted Wnt pathway components reveals unexpected complexity of the planarian amputation response. *Developmental Biology* 347:24-39.

Hendelberg J, Akesson B (1988). *Convolutriloba retrogemma* gen. et sp.n., a turbellarian (Acoela, Platyhelminthes) with reversed polarity of reproductive buds. *Fortschritte der Zoologie* 36: 321-327.

Hendelberg J, Akesson B (1991). Studies of the budding process in *Convolutriloba retrogemma* (Acoela, Platyhelminthes). *Hydrobiologia* 227:11-17.

Huang SM, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, Charlat O, Wiellette E, Yue Z, Wiessner S, Hild M, Xiaoying S, Wilson CJ, Mickanin C, Myer V, Fazal A, Tomlinson R, Serluca F, Shao W, Cheng H (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. *Nature* 461:614-620.

Hughes RN (1989). *A functional Biology of Clonal Animals*. London: Chapman and Hall.

Hyman JM, Firestone AJ, Heine VM, Zhao Y, Ocasio CA, Han K, Sun M, Rack PG, Sinha S, Wu JJ, Solow-Cordero DE, Jiang J, Rowitch DH, Chen JK, Scott MP (2009). Small-molecule inhibitors reveal multiple strategies for Hedgehog pathway blockade. *Proceedings of the National Academy of Sciences* 106: 14132-14137.

Iglesias M, Gomez-Skarmeta JL, Salo E, Adell T (2008) Silencing of Smed-beta-catenin1 generates radial-like hypercephalized planarians. *Development* 135:1215-1221.

Liu SY, Selck C, Friedrich B, Lutz R, Vila-Farre M, Dahl A, Brandl H, Lakshmanaperumal N, Henry I, Rink JC (2013). Reactivating head regrowth in a regeneration-deficient planarian species. *Nature* 500:81-84.

Martindale MQ (2005). The evolution of metazoan axial properties. *Nature Reviews Genetics* 6: 917-927.

Nusslein-Volhard C, Wieschaus E (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* 287: 795-801.

Pan A, Chang L, Nguyen A, James AW (2013). A review of hedgehog signaling in cranial bone development. *Frontiers in Physiology* 4:1-14.

Paps J, Baguna J, Riutort M (2009). Lophotrochozoa Internal Phylogeny: New Insights from an Up-to-Date Analysis of Nuclear Ribosomal Genes. *Proceedings of the Royal Society* 276: 1245-1254.

Petersen CP, Reddien PW (2008). *Smed-*β*catenin-1* is required for anteroposterior blastema polarity in planarian regeneration. *Science* 319:327-330.

Petersen CP, Reddien PW (2009). Wnt signaling and the polarity of the primary body axis. *Cell* 139: 1056-1068.

Rink JC, Gurley KA, Elliott SA, Alvarado AS (2009). Planarian Hh Signaling Regulates

Regeneration Polarity and Links Hh Pathway Evolution to Cilia. *Science* 326:1406-1410.

Ruiz-Trillo I, Riutort M, Littlewood DTJ, Herniou EA, Baguna J (1999). Acoel flatworms: earliest extant bilaterian metazoans, not member of Platyhelminthes. *Science* 283: 1919-1923.

Shannon III T, Achatz JG (2007). *Convolutriloba macropyga* sp. nov., an uncommonly fecund acoel (Acoelomorpha) discovered in tropical aquaria. *Zootaxa* 1525: 1-17.

Sikes JM, Bely AE (2010). Making heads from tails: development of a reversed anterior-posterior axis during budding in an acoel flatworm. *Developmental Biology* 338: 86-97.

Sikes JM, Bely AE (2008). Radical modification of the A-P axis and the evolution of asexual reproduction in *Convolutriloba* acoels. *Evolution and Development* 10: 619-631.

Sikes JM, Newmark PA (2013). Restoration of anterior regeneration in a planarian with limited regenerative ability. *Nature* 500:77-80.

Sinha S, Chen JK (2006). Purmorphamine activates the Hedgehog pathway by targeting Smoothened. *Nature Chemical Biology* 2:29-30.

Srivastava, M, Mazza-Curll KL, van Wolfswinkel JC, Reddien PW (2014). Whole-body acoel regeneration is controlled by Wnt and Bmp-Admp Signaling. *Current Biology* 24:1107-1113.

Umesono Y, Tasaki J, Nishimura Y, Hrouda M, Kawaguchi E, Yazawa S, Nishimura O, Hosoda K, Inoue T, Agata K (2013). The molecular logic for planarian regeneration along the

anterior-posterior axis. *Nature* 500:73-76.

Wang Y, Zhou Z, Walsh CT, McMahon AP (2009) Selective translocation of intracellular Smoothened to the primary cilium in response to Hedgehog pathway modulation. *Proceedings of the National Academy of Sciences* 106:2623-2628.

Yazawa S, Umesono Y, Hayashi T, Tarui H, Agata K (2009). Planarian Hedgehog/Patched establishes anterior-posterior polarity by regulating Wnt signaling. *Proceeds of the National Academy of Sciences of the United States of America* 106:22329-22334.

Zhang Q, Major MB, Takanashi S, Camp ND, Nishiya N, Peters EC, Ginsberg MH, Schultz PG, Moon RT, Ding S (2007). Small-molecule synergist of the Wnt/β-catenin signaling pathway. *Proceedings of the National Academy of Sciences* 104:7444-7448.